Synthesis and initial *in vitro* characterization of a new P2X7R radioligand [^{18}F]IUR-1602

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Abstract

The overexpression of P2X7R is associated with neuroinflammation and plays an important role in various neurodegenerative diseases. The [^{18}F]fluoropropyl derivative of GSK1482160, [^{18}F]IUR-1602, has been first prepared and examined as a new potential P2X7R radioligand. The reference standard IUR-1602 was synthesized from *tert*-butyl (*S*)-5-oxopyrrolidine-2-carboxylate, fluoropropylbromide, and 2-chloro-3-(trifluoromethyl)benzylamine with overall chemical yield 13% in three steps. The target tracer [^{18}F]IUR-1602 was synthesized from desmethyl-GSK1482160 with 3-[^{18}F]fluoropropyl tosylate, prepared from propane-1,3-diyl bis(4-methylbenzenesulfonate) and K[^{18}F]F/Kryptofix2.2.2, in two steps and isolated by HPLC combined with SPE in 2-7% decay corrected radiochemical yield. The radiochemical purity was >99%, and the molar activity at end of bombardment (EOB) was 74-370 GBq/μmol. The potency of IUR-1602 in comparison with GSK1482160 was determined by a radioligand competitive binding assay using [^{11}C]GSK1482160, and the binding affinity K_i values for IUR-1602 and GSK1482160 are 23.6 and 3.07 nM, respectively. The initial *in vitro* evaluation results, 8-fold less potency of [^{18}F]IUR-1602 compared to [^{11}C]GSK1482160, prevent further *in vivo* evaluation of [^{18}F]IUR-1602 in animals and human.

Keywords: [^{18}F]IUR-1602 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoropropyl)-5-oxopyrrolidine-2-carboxamide); Purinergic P2X7 receptor (P2X7R); Radiosynthesis; Competitive binding assay; Positron emission tomography (PET).

1. Introduction

The purinergic receptor P2X ligand-gated ion channel type 7 (P2X7R) is an adenosine triphosphate (ATP)-gated ion-channel, and P2X7R is a key player in inflammation (Lister et al., 2007). P2X7R is an emerging therapeutic target in central nervous system (CNS) diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), because the overexpression of P2X7R is associated with neuroinflammation (Sperlágh and Illes, 2014). P2X7R has become a novel molecular imaging target for neuroinflammation via biomedical imaging technique positron emission tomography (PET) (Tronel et al., 2017). Recently several radioligands targeting P2X7R have been developed and evaluated in animals, and the representative radioligands are shown in Figure 1 (Fantoni et al., 2017; Gao et al., 2015a; Han et al., 2017; Janssen et al., 2014, 2018; Jin et al., 2018; Ory et al., 2016; Territo et al., 2017). However, preclinical evaluation indicated some of these P2X7R radioligands have significant drawbacks like disadvantage of short half-life in clinical utility, limited blood-brain barrier (BBB) penetration and/or little brain uptake. Thus an ideal P2X7R radioligand that can be used in the clinical setting to study P2X7R expression levels in neurodegenerative disorders remains to be developed. In our previous work, we have developed and characterized [^{11}C]GSK1482160 as a P2X7R radioligand for neuroinflammation (Gao et al., 2015a; Territo et al., 2017), clinical evaluation of [^{11}C]GSK1482160 in health controls and patients are currently underway, and the estimation of radiation dosimetry for [^{11}C]GSK1482160 in normal human subjects has been reported (Green et al., 2018). Since the half-life ($t_{1/2}$) of radionuclide carbon-11 is only 20.4 min, it is attractive for us to develop derivatives of [^{11}C]GSK1482160 which can be labeled with the radionuclide fluorine-18 ($t_{1/2}$, 109.7 min), and a fluorine-18 ligand would be ideal for widespread

use. To this end a series of [^{18}F]fluoroalkyl derivatives of GSK1482160 will be considered as new potential P2X7R radioligands. The [^{18}F]fluoroethyl tosylate ([^{18}F]FEOTs) and [^{18}F]fluoropropyl tosylate ([^{18}F]FPOTs) are two most commonly used fluorine-18 labeled aliphatic building blocks for PET radioligand synthesis, and numerous [^{18}F]fluoroalkyl tracers have been synthesized by the replacement of a methyl group with a fluoroethyl (FE) or fluoropropyl (FP) group (van der Born et al., 2017). For instance, radiolabeled β-CIT-FE and β-CIT-FP have been proven to be superior to radiolabeled β-CIT for dopamine transporter (DAT) visualization (Günther et al., 1997; Zheng and Mulholland, 1996). In this ongoing study, we have recently reported the synthesis and preliminary biological evaluation of a new P2X7R radioligand, [^{18}F]fluoroethyl derivative of GSK1482160, [^{18}F]IUR-1601 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoroethyl)-5-oxopyrrolidine-2-carboxamide, Figure 1) (Gao et al., 2018), which retains the P2X7R affinity of [^{11}C]GSK1482160. Previous reports indicated DAT FE- and FP-derivative radioligands have very similar even superior biological activity to the parent radioligand (Abi-Dargham et al., 1996; Günther et al., 1997; Okada et al., 1998), and these results encourage us to develop [^{18}F]fluoropropyl derivative of GSK1482160, [^{18}F]IUR-1602 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoropropyl)-5-oxopyrrolidine-2-carboxamide, [^{18}F]**3a**, Figure 1). Furthermore, we wonder if the chemistry and biology of [^{18}F]fluoropropyl derivative of GSK1482160 could resemble those of [^{18}F]fluoroethyl derivative of GSK1482160. To this objective, herein we report the design, synthesis, radiolabeling and initial *in vitro* characterization of [^{18}F]IUR-1602 as a novel P2X7R radioligand.

Insert Figure 1 about here

2. Results and discussion

2.1. Chemistry

Like [^{18}F]IUR-1601, our initial synthetic strategy was to conduct an one-step radiosynthesis of [^{18}F]IUR-1602. The reference standard IUR-1602 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-fluoropropyl)-5-oxopyrrolidine-2-carboxamide, **3a**) and its corresponding precursor Cl-IUR-1602 ((*S*)-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-1-(2-chloropropyl)-5-oxopyrrolidine-2-carboxamide, **3b**) were synthesized as outlined in Scheme 1, according to the published methods (Gao et al., 2015a). *tert*-Butyl (*S*)-5-oxopyrrolidine-2-carboxylate was reacted with 1-bromo-2-fluoropropane (or 1-bromo-2-chloropropane) and NaH in *N,N*-dimethylformamide (DMF) to convert into amide *N*-alkylated product **1a** (or **1b**) in 44% and 41% yield, respectively. Subsequently, compound **1a** (or **1b**) was treated with trifluoroacetic acid (TFA) to give acid **2a** (or **2b**). Without further purification, compound **2a** (or **2b**) was undergone a coupling reaction with 2-chloro-3-(trifluoromethyl)benzylamine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) or 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as catalyst, affording IUR-1602 (or Cl-IUR-1602) in about 29% and 27% yield, respectively.

Insert Scheme 1 about here

Br-IUR-1602 was originally designed to be synthesized as the precursor for one-step [^{18}F]-labeling as shown in Scheme 2, because Br- is a better leaving group than Cl- for [^{18}F]-fluorination reaction (nucleophilic substitution). Starting from desmethyl-GSK1482160, we were unable to obtain the desired product Br-IUR-1602; instead, (*S*)-1-allyl-*N*-(2-chloro-3-(trifluoromethyl)benzyl)-5-oxopyrrolidine-2-carboxamide (allyl-IUR-1602, **4**) was isolated in 36% yield. Thus, Br-IUR-1602 appears unstable under these basic conditions, undergoing elimination to produce **4**.

Insert Scheme 2 about here

One-step radiosynthesis of [^{18}F]IUR-1602 was attempted as indicated in Scheme 3 using K[^{18}F]F/Kryptofix2.2.2 and acetonitrile as solvent at 100-110 ºC via a published method for [^{18}F]IUR-1601 (Gao et al., 2018). However, the Cl-IUR-1602 precursor failed to produce the radiolabeled product [^{18}F]IUR-1602. Then we conducted a model fluorination reaction without radioactivity at lower reaction temperature 80-100 ºC, which was monitored by analytical reverse-phase (RP) high performance liquid chromatography (HPLC). No IUR-1602 was detected, instead allyl-IUR-1602 was formed and detected by analytical RP HPLC, and confirmed by liquid chromatography-mass spectra (LC-MS), MS (ESI): $[M+H]^+$ 361; $[M-H]^-$ 359. The result was summarized in Scheme 4.

Insert Scheme 3 and Scheme 4 about here

A few other model reaction conditions were examined as shown in Scheme 5. The results suggested that at room temperature (RT) Cl-IUR-1602 is stable, but would not fluorinate to IUR-1602; IUR-1602 is stable at high temperature, but under strong base and high temperature conditions it would partially form allyl-IUR-1602 via elimination reaction as well. Thus we conclude the one-step radiosynthesis starting from chloro propyl- precursor is unlikely to be suitable for producing [^{18}F]IUR-1602.

Insert Scheme 5 about here

Therefore, we turned our efforts to a two-step radiosynthesis of [^{18}F]IUR-1602 using the precursors desmethyl-GSK1482160 and radiolabeled 3-[^{18}F]fluoropropyl tosylate (3-[^{18}F]F$CH_2CH_2CH_2$OTs) (Koivula et al., 2005) prepared from propane-1,3-diyl bis(4-methylbenzenesulfonate (Ts$OCH_2CH_2CH_2$OTs) and K[^{18}F]F/Kryptofix2.2.2. Prior to the radiosynthesis, several model reactions in small scale (0.5-1.0 mg) without radioactivity were performed to investigate the base and reaction temperature effects on the fluoropropylation of cyclic amide of the desmethyl-GSK1482160 precursor, to maximize the IUR-1602 yield and to minimize the allyl-IUR-1602 yield, as depicted in Scheme 6. All reactions were monitored by analytical RP HPLC. Based on the results we obtained, we believed that the cyclic amide is more easily deprotonated and alkylated than side chain amide; amide alkylation is a tough reaction and requires strong base and high reaction temperature conditions, the best base is 2 M NaOH, and the best reaction temperature is 100-110 ºC; and amide fluoropropylation reaction was quickly followed by an elimination reaction to form allyl-IUR-1602 under strong reaction conditions.

Further optimization of the reaction conditions provided the best method for two-step radiosynthesis of [^{18}F]IUR-1602.

Insert Scheme 6 about here

The corresponding two-step radiosynthesis of [^{18}F]IUR-1602 is shown in Scheme 7. $TsOCH_2CH_2CH_2OTs$ was labeled with K[^{18}F]F/Kryptofix2.2.2 to form a radiolabeled precursor 3-[^{18}F]$FCH_2CH_2CH_2OTs$, which was simply purified by a C18 Plus Sep-Pak cartridge. Then 3-[^{18}F]$FCH_2CH_2CH_2OTs$ was reacted with desmethyl-GSK1482160 at 100-110 ºC under basic condition (2 M NaOH) for 20 min to give the target tracer [^{18}F]IUR-1602. This is a two-step two-pot radiosynthesis. [^{18}F]IUR-1602 was isolated by the semi-preparative RP HPLC (C18 column), and then purified from the HPLC mobile phase with solid-phase extraction (SPE) using a C18 Plus Sep-Pak cartridge. The radiosynthesis was performed in a self-designed automated multi-purpose [^{18}F]-radiosynthesis module (Gao et al., 2018; Wang et al., 2012). This radiosynthesis module facilitated the overall design of the reaction, purification and reformulation capabilities in a fashion suitable for adaptation to preparation of human doses. As we previously indicated, [^{18}F]fluoropropylation of amide requires high reaction temperature and strong base; in addition, the labeled product [^{18}F]IUR-1602 is unstable under the strong reaction conditions, partially converting to allyl-IUR-1602 by elimination reaction. The two-step radiosynthesis was a somewhat “hit-and-run” radiosynthesis, which resulted in low radiochemical yield. The overall radiochemical yield for two-step radiosynthesis of [^{18}F]IUR-1602 was 2-7% decay corrected to end of bombardment (EOB) based on H[^{18}F]F. The overall synthesis time was ~2 h from EOB. The molar activity was 74-370 GBq/μmol at EOB,

determined by a "spike" method using analytical HPLC at end of synthesis (EOS). The general methods to increase the molar activity have been described in our previous works (Gao et al., 2015b, 2018; Wang et al., 2011, 2014), which include rinsing the H[^{18}F]F delivery line with H_2[^{18}O]O prior to the H[^{18}F]F production, eliminating QMA Sep-Pak cartridge to trap/release the H[^{18}F]F, and using optimized beam current and beam time to produce the H[^{18}F]F. To facilitate the potential application of [^{18}F]IUR-1602 in animals, it is necessary to improve its radiochemical yield. However, the chemistry nature of [^{18}F]IUR-1602 radiosynthesis (two-step "hit-and-run") does not provide much room for the improvement. Thus, the further radiosynthesis optimization of [^{18}F]IUR-1602 will focus on the hardware and software modification of our existing home-built [^{18}F]-radiosynthesis module, such as enlarging the volume of reaction V-vial and revising the computer-controlled synthesis sequences, to minimize radioactivity loss.

Insert Scheme 7 about here

Chemical purity and radiochemical purity were determined by analytical HPLC (Gao et al., 2015b; Wang et al., 2011, 2014). The chemical purity of the precursor and reference standard was >95% determined by RP HPLC through UV flow detector. The radiochemical purity of the target tracer was >99% determined by radio-HPLC through γ-ray (PIN diode) flow detector.

The analytical RP HPLC system used to monitor all organic synthetic and radiosynthetic reactions included a Prodigy (Phenomenex) 5 μm C-18 column, 4.6 × 250 mm, a gradient mobile phase (40-80%) CH_3CN/3 mM $HCOONH_4$, flow rate 1.8 mL/min; UV (270 nm) and γ-ray (PIN

diode) flow detectors. Table 1 lists the retention time (t_R) data determined by this HPLC system for the compounds involved in the synthetic reactions, and their corresponding LogP and cLogP values calculated from ChemDraw Professional 15.1.

Insert Table 1 about here

GSK1482160, desmethyl-GSK1482160 and [^{11}C]GSK1482160 were synthesized in our previous work (Gao et al., 2015a).

2.2. In vitro characterization

The initial *in vitro* characterization of IUR-1602 in comparison with IUR-1601 and the known P2X7R ligand GSK1482160 was performed by a radioligand competitive binding assay using [^{11}C]GSK1482160 following the literature method (Gao et al., 2017, 2018; Sihver et al., 1997; Territo, et al., 2017; Wang et al., 2017). The results are shown in Figure 2. [^{11}C]GSK1482160 was used as the radioligand, GSK1482160 and buffer were used as a positive control and a negative control, respectively. All data were analyzed by GraphPad Prism 7 software using a curve fitting equation to fit the K_i. The binding affinity K_i values (nM) for IUR-1602, IUR-1601 and GSK1482160 are 23.6 ± 0.96, 3.73 ± 0.30 and 3.07 ± 0.24 (n =3), respectively. Compared to IUR-1601 and GSK1482160, there is ~8-fold decrease of Ki value of IUR-1602. Our new findings suggest FE-derivative IUR-1601 had similar P2X7R binding affinity to the parent compound GSK1482160, and FP-derivative IUR-1602 showed lower binding affinity toward the P2X7R than that of GSK1482160, which are different with the literature results (Abi-Dargham et

al., 1996; Günther et al., 1997; Okada et al., 1998). The K_i value of IUR-1602 (23.6 nM) is still in low nanomolar range, making [^{18}F]IUR-1602 as a suitable candidate P2X7R radioligand for further biological study.

Insert Figure 2 about here

3. Experimental

3.1. General

All commercial reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific, and used without further purification. Melting points were determined on a MEL-TEMP II capillary tube apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 500 MHz NMR Fourier transform spectrometer at 500 and 125 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to an internal standard tetramethylsilane (TMS, δ 0.0) (^{1}H NMR) and to the solvent signal (^{13}C NMR), and coupling constants (J) are reported in hertz (Hz). LC-MS analysis was performed on an Agilent system, consisting of an 1100 series HPLC connected to a diode array detector and a 1946D mass spectrometer configured for positive-ion/negative-ion electrospray ionization. The high resolution mass spectra (HRMS) were obtained using a Waters/Micromass LCT Classic spectrometer. Chromatographic solvent proportions are indicated as volume: volume ratio. Thin-layer chromatography (TLC) was run using Analtech silica gel GF uniplates (5 × 10 cm^2). Plates

were visualized under UV light. Normal phase flash column chromatography was carried out on EM Science silica gel 60 (230-400 mesh) with a forced flow of the indicated solvent system in the proportions described below. All moisture- and air-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. C18 Plus Sep-Pak cartridges were obtained from Waters Corporation (Milford, MA). Sterile Millex-FG 0.2 μm filter units were obtained from Millipore Corporation (Bedford, MA). [^{11}C]GSK1482160 is a routine radiotracer produced in our PET radiochemistry facility.

*3.2. tert-Butyl (S)-1-(3-fluoropropyl)-5-oxopyrrolidine-2-carboxylate (**1a**)*

tert-Butyl (*S*)-5-oxopyrrolidine-2-carboxylate (3.74 g, 20 mmol) was dissolved in DMF (30 mL) and cooled to 0 °C. Sodium hydride (1.20 g of 60% suspension in mineral oil, 30 mmol) was added to reaction mixture and stirred for 0.5 h. Then 1-bromo-3-fluoropropane (5.60 g, 40 mmol) was added slowly, the reaction mixture was allowed to warm to RT and stirred for overnight. The solvent was evaporated in vacuo, and water was added, subsequently the aqueous layer was extracted with EtOAc (100 mL × 3), and the combined organic layers were dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by column chromatography on silica gel with eluent (1:99 MeOH/CH_2Cl_2) to yield colorless oily product **1a** (2.16 g, 44%), R_f = 0.78 (1:15 MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H, 3 × CH_3), 1.88-1.95 (m, 2H, CH_2), 2.03-2.07 (m, 1H, CH), 2.26-2.37 (m, 2H, CH_2), 2.47-2.49 (m, 1H, CH), 3.12-3.15 (m, 1H, CH), 3.68-3.73 (m, 1H, CH), 4.07 (dd, J = 3.0, 9.0 Hz, 1H, CH), 4.41-4.44 (m, 1H, CH), 4.50-4.53 (m, 1H, CH). MS (ESI): 246 ([M+H]$^+$, 100%).

*3.3. tert-Butyl (S)-1-(3-chloropropyl)-5-oxopyrrolidine-2-carboxylate (**1b**)*

Compound **1b** was prepared using the same procedure as described for the synthesis of **1a** by substituting 1-bromo-3-chloropropane for 1-bromo-3-fluoropropane. Colorless oil, yield 41%, R_f = 0.77 (1:15 MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.49 (s, 9H, 3 × CH_3), 1.95-1.99 (m, 1H, CH), 2.00-2.10 (m, 2H, CH_2), 2.26-2.36 (m, 2H, CH_2), 2.47-2.50 (m, 1H, CH), 3.16-3.21 (m, 1H, CH), 3.51- 3.57 (m, 2H, CH_2), 3.64-3.69 (m, 1H, CH), 4.06 (dd, J = 3.0, 8.5 Hz, 1H, CH). MS (ESI): 262 ($[M+H]^+$, 100%).

*3.4. (S)-1-(3-Fluoropropyl)-5-oxopyrrolidine-2-carboxylic acid (**2a**)*

A solution of compound **1a** (578 mg, 2.5 mmol) in dichloromethane was added into TFA (1.2 mL) at RT, the reaction mixture was then stirred for 36 h. Subsequently the reaction mixture was evaporated to dry under reduced pressure. Toluene was added to residue, and this was in turn also evaporated to give yellowish oily product **2a**, which was used for next step without further purification.

*3.5. (S)-1-(3-Chloropropyl)-5-oxopyrrolidine-2-carboxylic acid (**2b**)*

Compound **2b** was prepared using the same procedure as described for the synthesis of **2a** by substituting **1b** for **1a**.

*3.6. (S)-N-(2-Chloro-3-(trifluoromethyl)benzyl)-1-(3-fluoropropyl)-5-oxopyrrolidine-2-carboxamide (IUR-1602, **3a**)*

Compound **2a** (473 mg, 2.5 mmol) and 2-chloro-3-(trifluoromethyl)benzylamine (419 mg, 2.0 mmol) were mixed with EDAC (480 mg, 2.5 mmol) and 1-hydrobenzotrizole (HOBt, 338 mg, 2.5 mmol) in dry dichloromethane (60 mL) and the reaction mixture was stirred for overnight at RT. Then the resultant mixture was washed with 2 N HCl (50 mL) and saturated aqueous sodium hydrogen carbonate (40 mL). The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with eluent (2:98 MeOH/CH_2Cl_2) to afford a white solid product **3a** (220 mg, 29%), R_f = 0.37 (1:19 MeOH/CH_2Cl_2), mp 128-130 °C. ^{1}H NMR ($CDCl_3$): δ 1.84-1.89 (m, 2H, CH_2), 2.04-2.07 (m, 1H, CH), 2.30-2.35 (m, 2H, CH_2), 2.47-2.50 (m, 1H, CH), 3.00-3.06 (m, 1H, CH), 3.69-3.75 (m, 1H, CH), 4.11 (dd, J = 3.5, 8.5 Hz, 1H, CH), 4.36 (dt, J = 1.5, 5.5 Hz, 1H, CH), 4.45 (dt, J = 1.5, 5.5 Hz, 1H, CH), 4.61 (d, J = 6.0 Hz, 2H, CH_2Ph), 6.69 (t, J = 5.5 Hz, 1H, CONH), 7.36 (t, J = 8.0 Hz, 1H, Ph-H), 7.58 (d, J = 8.0 Hz, 1H, Ph-H), 7.65 (dd, J = 1.5, 8.0 Hz, 1H, Ph-H). ^{13}C NMR ($CDCl_3$): δ 23.92, 28.24 (d, $J_{C\text{-}F}$ = 20 Hz), 29.72, 39.22 (d, $J_{C\text{-}F}$ = 5.0 Hz), 41.92, 62.00, 82.33 (d, $J_{C\text{-}F}$ = 163.75 Hz, CH_2F), 121.81 (q, $J_{C\text{-}F}$ = 271.75 Hz, CF_3), 127.09, 127.35 (q, $J_{C\text{-}F}$ = 5.71 Hz), 129.29 (q, $J_{C\text{-}F}$ = 31.38 Hz), 131.82, 133.99, 137.73, 171.74, 176.09. MS (ESI): 381 ($[M+H]^+$, 100%); MS (ESI): 379 ($[M-H]^-$, 50%). HRMS (ESI): calcd for $C_{16}H_{18}N_2O_2ClF_4$ ($[M+H]^+$) 381.0987, found 381.0992.

*3.7. (S)-N-(2-Chloro-3-(trifluoromethyl)benzyl)-1-(3-chloropropyl)-5-oxopyrrolidine-2-carboxamide (Cl-IUR-1602, **3b**)*

Compound **3b** was prepared using the same procedure as described for the synthesis of **3a** by substituting **2b** for **2a**. White solid, yield 27%, R_f = 0.36 (1:19 MeOH/CH_2Cl_2), mp 121-123 °C. ^{1}H NMR ($CDCl_3$): δ 1.90-1.94 (m, 1H, CH), 1.97-2.08 (m, 2H, CH_2), 2.32-2.37 (m, 2H, CH_2), 2.49-2.53 (m, 1H, CH), 3.04-3.10 (m, 1H, CH), 3.47-2.51 (m, 2H, CH_2), 3.67-3.72 (m, 1H, CH), 4.09 (dd, J = 3.5, 8.5 Hz, 1H, CH), 4.62 (d, J = 6.0 Hz, 2H, CH_2Ph), 6.48 (t, J = 5.5 Hz, 1H, CONH), 7.37 (t, J = 8.0 Hz, 1H, Ph-H), 7.60 (d, J = 8.0 Hz, 1H, Ph-H), 7.66 (dd, J = 1.0, 8.0 Hz, 1H, Ph-H). MS (ESI): 397 ([M+H]$^+$, 100%); MS (ESI): 395 ([M-H]$^-$, 80%).

*3.8. (S)-1-Allyl-N-(2-chloro-3-(trifluoromethyl)benzyl)-5-oxopyrrolidine-2-carboxamide (allyl-IUR-1602, **4**)*

Desmethyl-GSK1482160 (320 mg, 1.0 mmol) was dissolved in DMF (20 mL) and cooled to 0 °C. Sodium hydride (80 mg of 60% suspension in mineral oil, 2.0 mmol) was added to reaction mixture and stirred for 30 min. Then 1,3-dibromopropane (505 mg, 2.5 mmol) was added slowly, the reaction mixture was allowed to warm to RT, and stirred for overnight. The solvent was evaporated in vacuo, and water was added, subsequently the aqueous layer was extracted with EtOAc (50 mL × 3), and dried over $MgSO_4$. The combined organic layer was concentrated, and the residue was purified by column chromatography on silica gel with eluent (3:97 MeOH/CH_2Cl_2) to yield a white solid product **4** (130 mg, 36%), R_f = 0.71 (1:19 MeOH/CH_2Cl_2), mp 131-133 °C. ^{1}H NMR ($CDCl_3$): δ 2.03-2.06 (m, 1H, CH), 2.31-2.39 (m, 2H, CH_2), 2.51-2.54 (m, 1H, CH), 3.39 (dd, J = 1.0, 7.5 Hz, 1H, CH), 4.03 (dd, J = 3.5, 9.0 Hz, 1H, CH), 4.27 (dd, J = 1.5, 4.0 Hz, 1H, CH), 4.60 (dd, J = 1.5, 6.0 Hz, 2H, CH_2Ph), 5.02 (dd, J = 1.5, 17.0 Hz, 1H, =CH), 5.09 (dd, J = 1.0, 10.0 Hz, 1H, =CH), 5.65-5.68 (m, 1H, =CH), 6.40 (br s, 1H, CONH),

7.37 (t, J = 8.0 Hz, 1H, Ph-H), 7.60 (d, J = 7.5 Hz, 1H, Ph-H), 7.66 (dd, J = 1.0, 8.0 Hz, 1H, Ph-H). MS (ESI): 361 ($[M+H]^+$, 100%); MS (ESI): 359 ($[M-H]^-$, 100%).

*3.9. (S)-N-(2-Chloro-3-(trifluoromethyl)benzyl)-1-(2-[^{18}F]fluoropropyl)-5-oxopyrrolidine-2-carboxamide ([^{18}F]IUR-1602, [^{18}F]**3a**)*

No-carrier-added (NCA) aqueous H[^{18}F]F was produced by ^{18}O(p,n)^{18}F nuclear reaction using a Siemens Eclipse RDS-111 cyclotron by irradiation of $H_2{}^{18}O$ (2.5 mL). H[^{18}F]F (~37 GBq) in [^{18}O]water plus 0.1 mL K_2CO_3 solution (1.7 mg) and Kryptofix 2.2.2 (10 mg) in 1.0 mL CH_3CN with additional 1 mL CH_3CN were placed in the fluorination reaction vial (10-mL V-vial) and repeated azeotropic distillation (17 min) was performed at 110 °C to remove water and to form the anhydrous K[^{18}F]F-Kryptofix 2.2.2 complex. $TsOCH_2CH_2CH_2OTs$ (15.4 mg, 4.0 mmol) dissolved in CH_3CN (1.0 mL) was introduced to the reaction vessel and heated at 100-110 °C for 20 min to affect radiofluorination. After cooling to 80 °C, the reaction mixture 3-[^{18}F]$FCH_2CH_2CH_2OTs$ was diluted with 3.0 mL H_2O, passed through a C18 Plus Sep-Pak cartridge, washed with H_2O (2 mL × 3), eluted with CH_3CN (2 mL × 2), transferred to a 10-mL V-vial, and repeated azeotropic distillation (17 min) was performed at 110 °C to remove water and to form the anhydrous 3-[^{18}F]$FCH_2CH_2CH_2OTs$. The precursor desmethyl-GSK1482160 (6.4 mg, 2.0 mmol) and 2 M NaOH (10 μL) in CH_3CN (1.0 mL) was introduced to the reaction vessel. The mixture was heated to 100-110 °C for 20 min, cooled to 80 °C, and then quenched with H_2O (2.0 mL). The mixture was injected onto the semi-preparative HPLC column with 3 mL injection loop for purification, using a YMC-Pack ODS-A, S-5 μm, 12 nm, 10×250 mm C18 (reverse phase) column; 40% CH_3CN:60% 3 mM $HCOONH_4$ mobile phase; 5.0 mL/min flow

rate; UV (270 nm) and γ-ray (PIN diode) flow detectors. The product fraction was collected in a recovery vial containing 35 mL water. The diluted tracer solution was then passed through a C-18 Plus Sep-Pak cartridge, and washed with water (5 mL × 5). At the same time, the HPLC mobile phase was automatically switched from 40% CH_3CN:60% 3 mM $HCOONH_4$ to 70% CH_3CN/30% H_2O to elute high lipophilic unreacted 3-[^{18}F]$FCH_2CH_2CH_2OTs$ and $TsOCH_2CH_2CH_2OTs$ from the HPLC column. The Sep-Pak cartridge was eluted with EtOH (1 mL × 2) to release [^{18}F]IUR-1602, followed by saline (10 mL). The eluted product was then sterile-filtered through a Millex-FG 0.2 μm membrane into a sterile vial. Total radioactivity was assayed and total volume was noted for tracer dose dispensing. Retention times in the semi-preparative HPLC system were: t_R desmethyl-GSK1482160 = 6.68 min, t_R allyl-IUR-1602 = 13.37 min, t_R IUR-1602 = 12.12 min, t_R [^{18}F]IUR-1601 = 12.25 min, t_R 3-$FCH_2CH_2CH_2OTs$ = 16.70 min, t_R 3-[^{18}F]$FCH_2CH_2CH_2OTs$ = 16.85 min, and t_R $TsOCH_2CH_2CH_2OTs$ = 19.87 min. Retention times in the analytical HPLC system were: t_R IUR-1602 = 4.69 min and t_R [^{18}F]IUR-1602 = 4.74 min. The decay corrected radiochemical yields of [^{18}F]IUR-1602 were 2-7%.

3.10. Cell culture and membrane preparation

HEK293 cells expressing human recombinant P2X7 receptor (hP2X7R) were obtained from B'SYS GmbH and cultured according to the supplier's procedure. Cells were grown to 80% confluency and then rinsed with phosphate-buffered saline (PBS), detached with trypsin, and harvested. Cell pellets were obtained by centrifugation at 200 *g* for 5 min at 4 °C. Collected cell pellets were frozen at −80 °C until membrane preparation. For the membrane preparation, pellets from 10 T225 flasks were homogenized in 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic

acid (EDTA), and 140 mM NaCl at pH 7.4 and 4 °C. The homogenate was then centrifuged at 48,000 *g* for 20 min at 4 °C, gently rinsed with deionized water, and then resuspended in 50 mM Tris-HCl at pH 7.4 and 4 °C. This homogenate was then centrifuged as before, with the resulting pellet being homogenized in 50 mM Tris-HCl at pH 7.4. Total protein concentration was determined via the Bradford protein assay (Bio-Rad). Aliquots were stored in cryovials at −80 °C until the day of assay.

3.11. [^{11}C]GSK1482160 standards

For each experiment, the highest concentration of radioactivity used was diluted twice in assay buffer, followed by eleven 2-fold dilutions. Twenty microliters of each dilution were then spotted onto a GF/B UniFilter-96 plate (Perkin-Elmer) and allowed to air-dry until the end of the experiment. An additional 20 μL of each dilution were added to a scintillation vial containing 7 mL of Optiphase Hisafe 3 (Perkin-Elmer) and counted on an LS6000 scintillation counter (Beckman). Aliquots of the working concentrations of radioactivity used on each day were counted in the same manner.

3.12. Radioligand competitive binding assay

For competitive binding assays, cell membrane preparation (0.054 mg of protein/mL of assay medium) was incubated with 11 compound concentrations over a six log unit range, with 5 nM [^{11}C]GSK1482160 in assay buffer (50 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumen (BSA)) . Triplicate determinations were done at each concentration of test compound.

GSK1482160 was used to determine non-specific binding. Assays were incubated at 22 °C for 30 min. For termination of the binding reaction, the samples were filtered onto GF/B UniFilter plates that had been presoaked in 0.5% polyethyleneimine for 30 min using a UniFilter-96 cell harvester. The plates were washed 5 times with ice-cold saline, dried under a vacuum, and exposed to a TR2025 phosphor screen for 20–60 min. Phosphor screens were then read on a Typhoon FLA-7000IP (GE Healthcare) along with [^{11}C]-calibration standards. CPMs (counts per minute) were determined by calibrating the image to the CPMs in the calibration standards via MCID analysis Software. Data was analyzed with Prism 7 (GraphPad Software Inc.) to determine K_i values.

4. Conclusion

In summary, the chemistry of [^{18}F]IUR-1602 is highly similar to the chemistry of [^{18}F]IUR-1601. Synthetic routes with reasonable to high yields have been developed to produce the reference standard IUR-1602 and its corresponding precursors for labeling. One-step and two-step radiosyntheses of [^{18}F]IUR-1602 have been studied. Various model reactions were designed to investigate the reasons that caused one-step synthesis of [^{18}F]IUR-1602 to fail and two-step synthesis of [^{18}F]IUR-1602 with low radiochemical yield. A new P2X7R radioligand [^{18}F]IUR-1602 has been successfully radiosynthesized. The initial *in vitro* evaluation results suggest [^{18}F]IUR-1602 remained low nM grade P2X7R affinity, but 8-fold decrease compared to [^{18}F]IUR-1601 and [^{11}C]GSK1482160; and prevent further *in vivo* evaluation of [^{18}F]IUR-1602

in animals and human. This new biology finding provides an example that FP-derivate radioligand does not always have similar or superior binding affinity to the parent radioligand.

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Conflict of interest statement

The authors declare that they have no conflict of interest relevant to this article.

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Table 1. t_R, LogP and cLogP of IUR-1601 and its analogs, $TsOCH_2CH_2CH_2OTs$ and $FCH_2CH_2CH_2OTs$.

Compound	LogP	cLogP	tR (min)
GSK1482160	1.88	2.26	N/D*
Desmethyl-GSK1482160	1.64	1.59	3.46
IUR-1602	2.17	2.74	4.69
Cl-IUR-1602	2.69	2.95	5.70
Allyl-IUR-1602	2.57	2.81	4.80
$TsOCH_2CH_2CH_2OTs$	3.75	3.22	>15.00**
$FCH_2CH_2CH_2OTs$	2.24	1.80	8.19

*N/D, Not determined.

** t_R of $TsOCH_2CH_2CH_2OTs$ is much longer than the t_R of radiolabeled product [^{18}F]IUR-1602 in semi-preparative HPLC column, and it will be eluted from the column using high lipophilic 70% CH_3CN/30% H_2O mobile phase after [^{18}F]IUR-1602 fraction collection.

Figure and Scheme Legends

Figure 1. Radioligands for imaging of P2X7R.

Figure 2. The result of the competitive binding assay of IUR-1602 in comparison with IUR-1601 and GSK1482160.

Scheme 1. Synthesis of IUR-1602 (**3a**) and its precursor Cl-IUR-1602 (**3b**). Reaction conditions and regents: (i) $BrCH_2CH_2X$, NaH, DMF; 44% and 41%. (ii) TFA, CH_2Cl_2. (iii) EDAC, HOBt, CH_2Cl_2, 29% and 27%.

Scheme 2. Synthesis of Br-IUR-1602 failed. Instead, Allyl-IUR-1602 (**4**) was obtained in 36% yield.

Scheme 3. One-step radiosynthesis of [^{18}F]IUR-1602 failed.

Scheme 4. Model fluorination reaction at 80-100 ºC.

Scheme 5. Model fluorination reaction and stability of Cl-IUR-1602 and IUR-1602.

Scheme 6. Base and reaction temperature effects on the fluoropropylation of cyclic amide of the desmethyl-GSK1482160.

Scheme 7. Two-step radiosynthesis of [^{18}F]IUR-1602.

Figure 1.

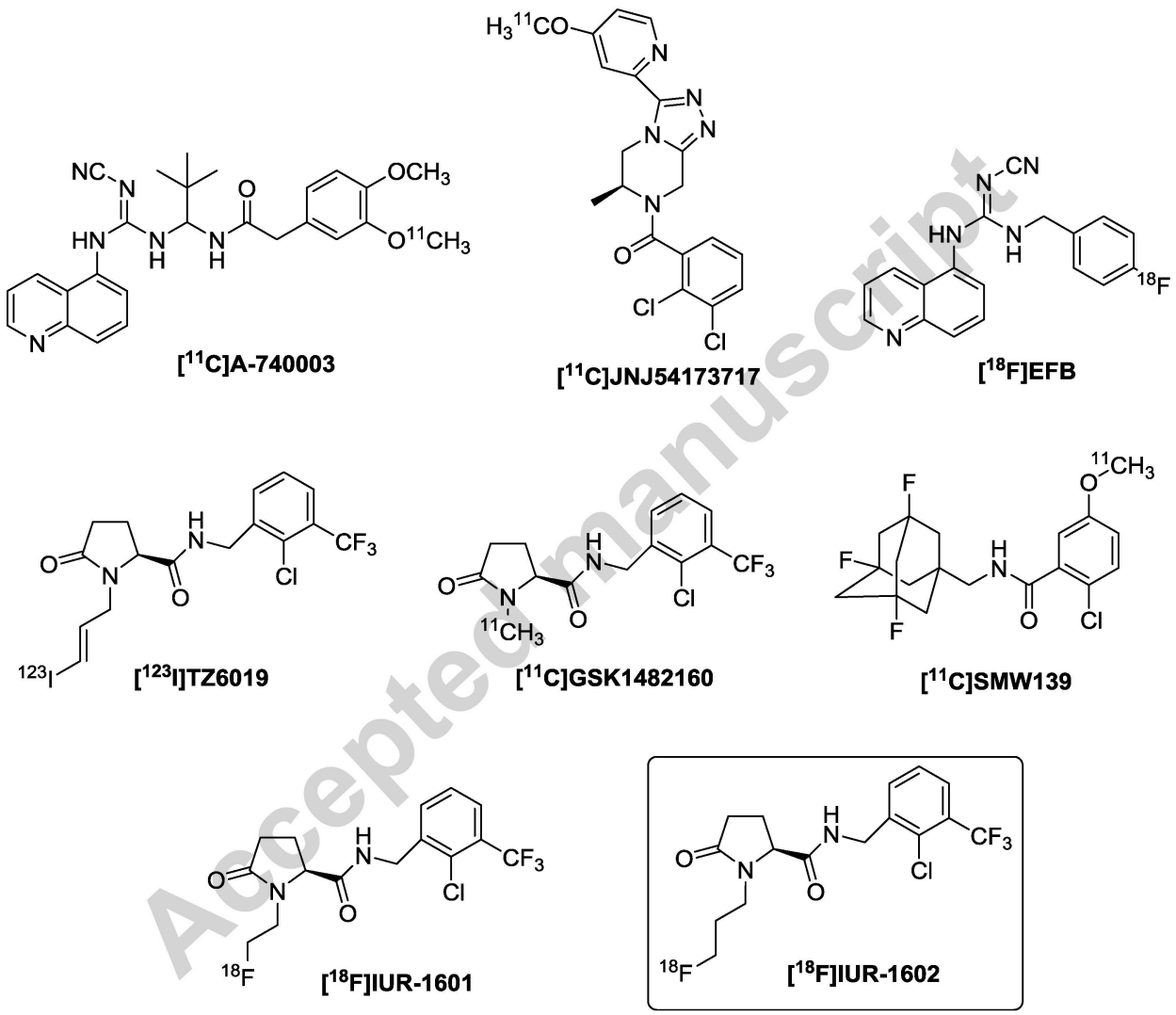
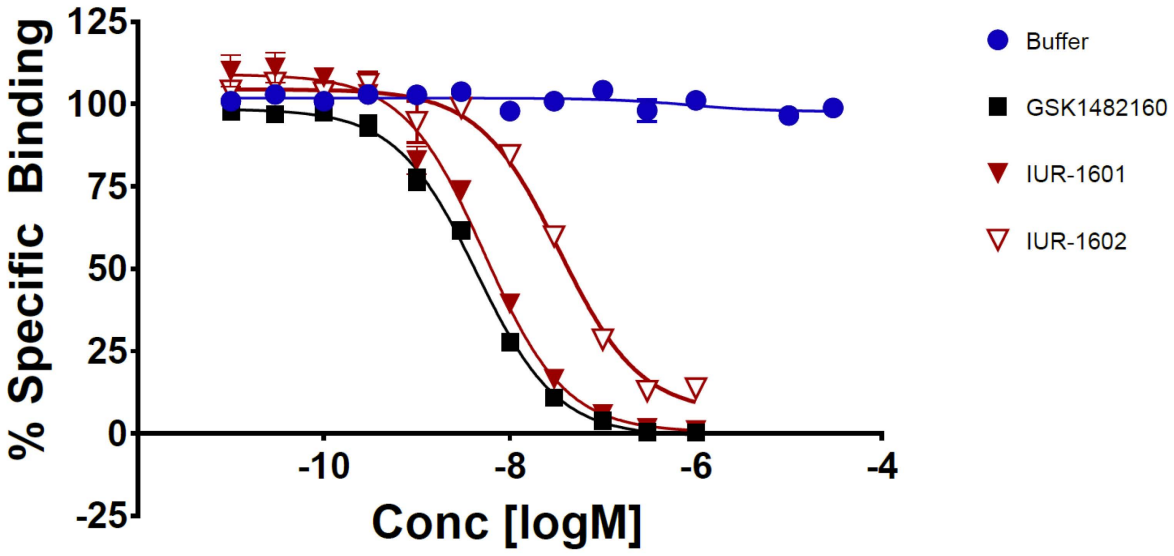
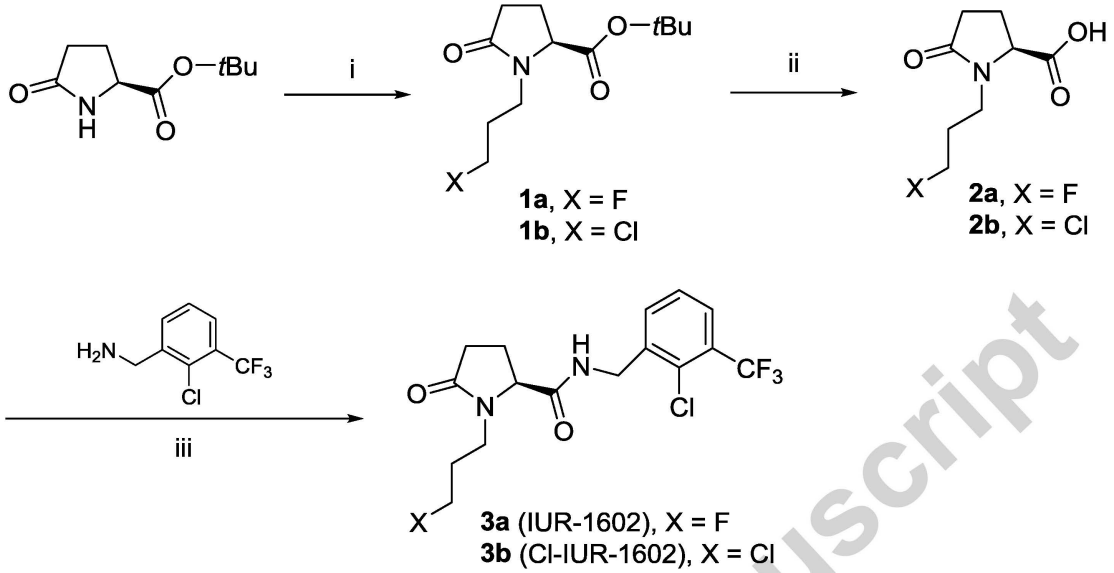


Figure 2.

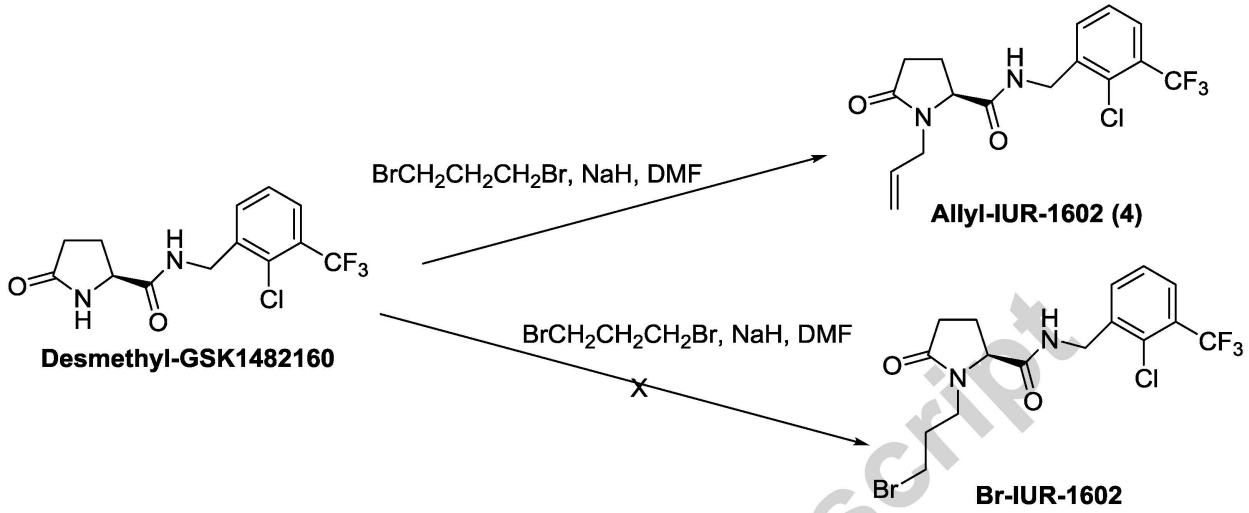


Compound	GSK1482160	IUR-1601	IUR-1602
K_i (nM)	3.07 ± 0.24	3.73 ± 0.30	23.6 ± 0.96

Scheme 1.



Scheme 2.



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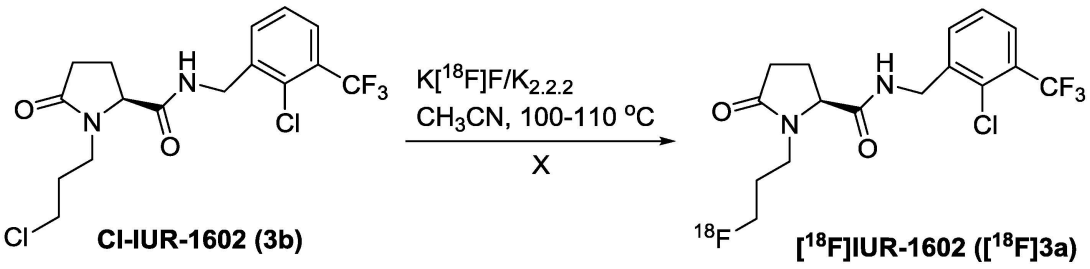
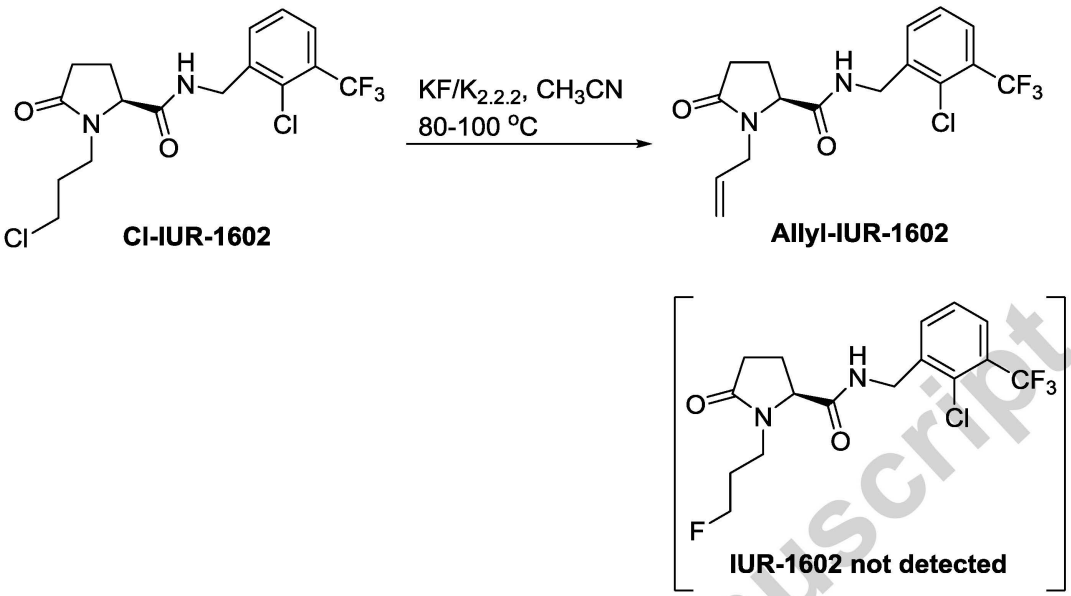


Scheme 4.



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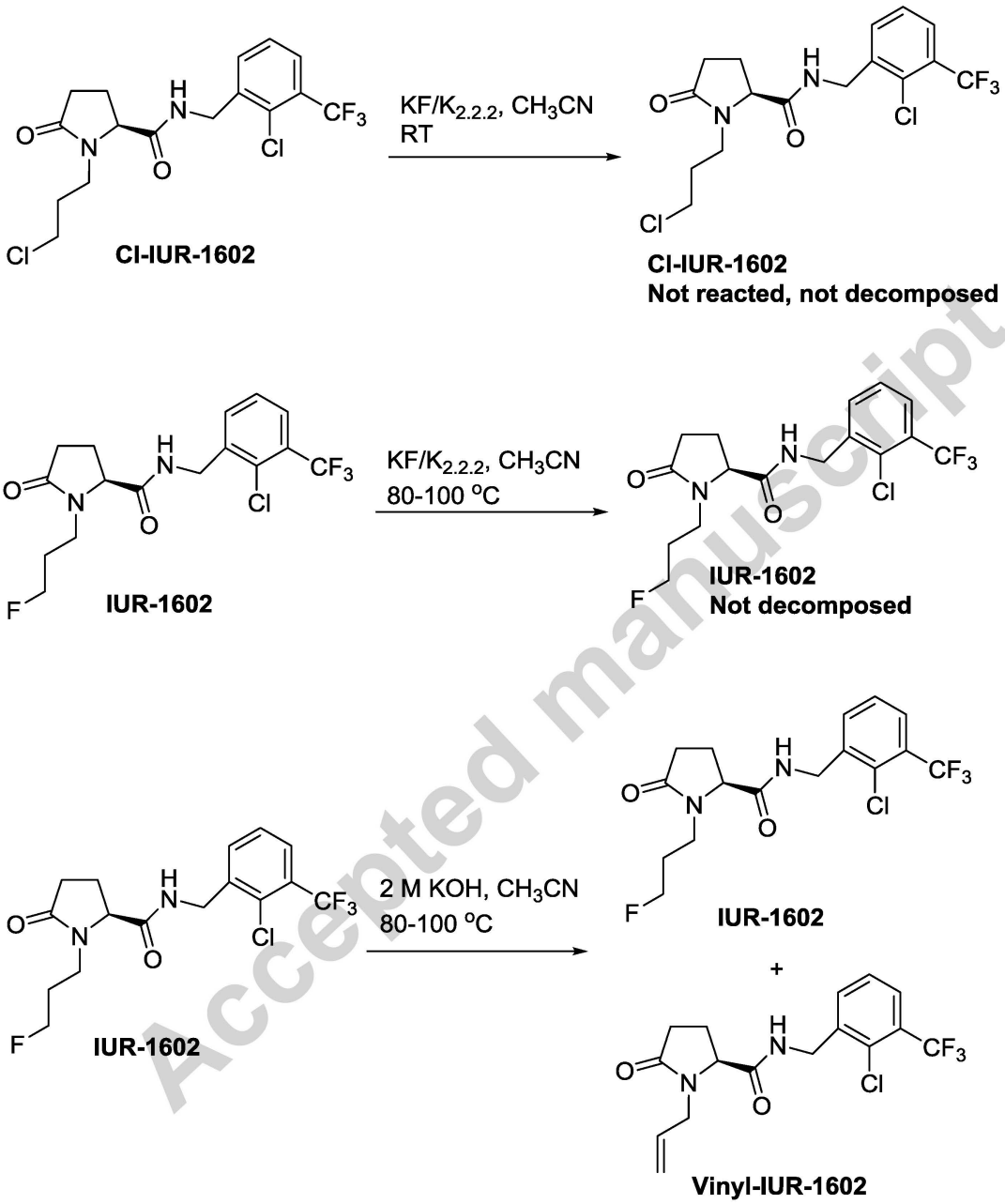

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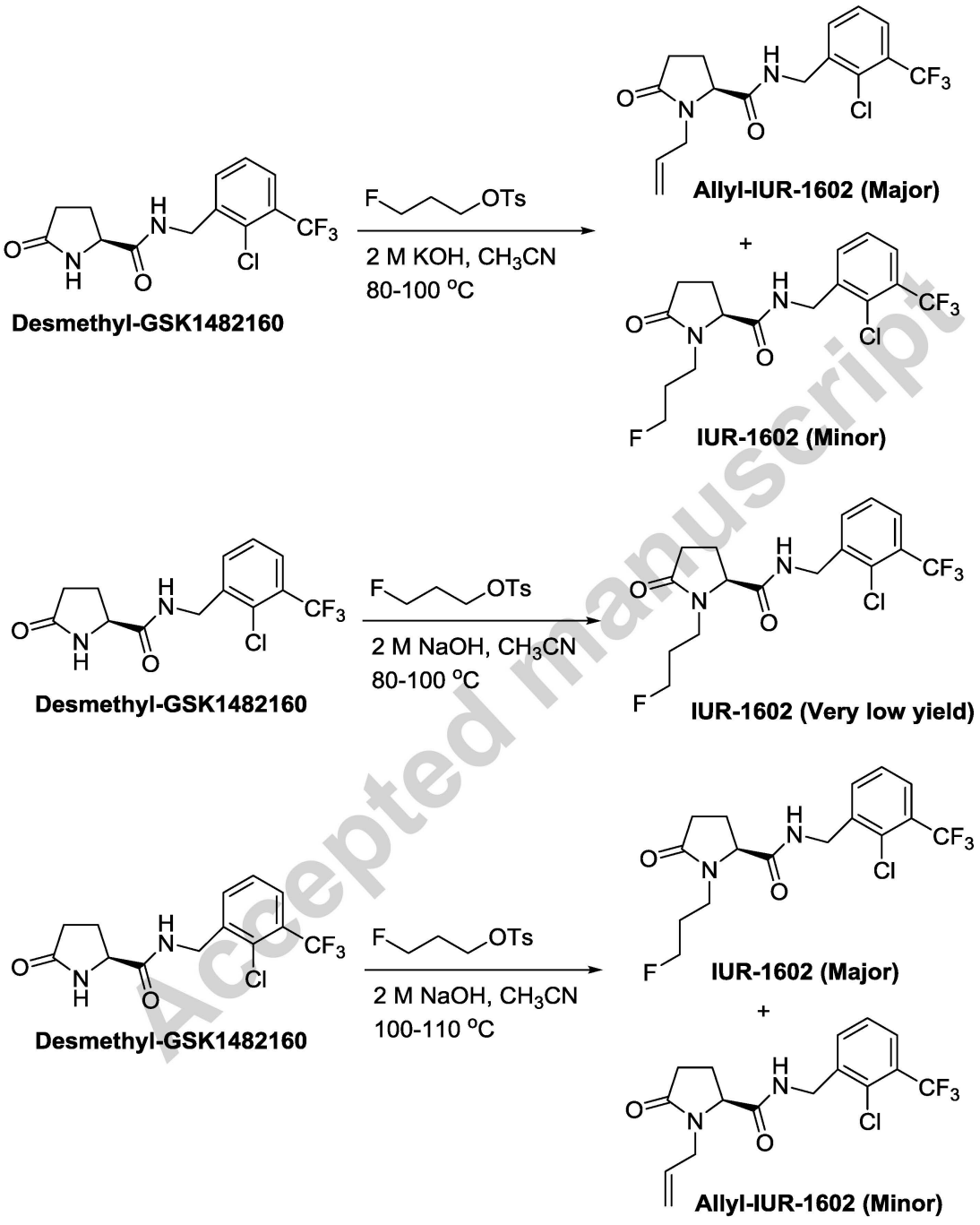
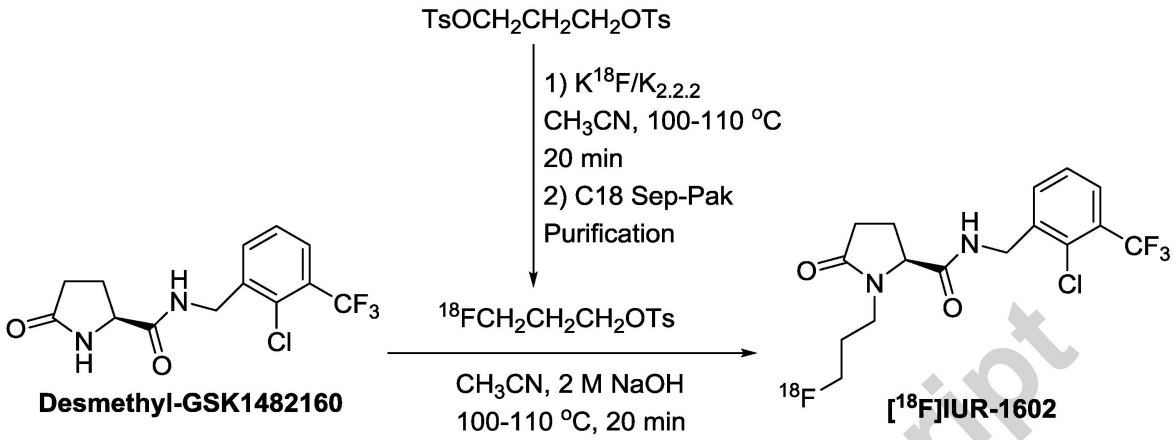


Scheme 7.



Highlights

- A new P2X7R radioligand [^{18}F]IUR-1602 was synthesized.
- A 2-step 2-pot fully automated [^{18}F]-radiosynthesis for [^{18}F]IUR-1602 was developed.
- A semi-preparative RP HPLC-SPE technique was employed in radiosynthesis.
- K_i value of IUR-1602 was 23.6 ± 0.96 nM.